ADENOVIRAL VA1 POL III EXPRESSION
SYSTEM FOR RNA EXPRESSION

[0001]   This application claims priority to co-pending U.S. Provisional Application Serial No. 60/399,397, filed July 31, 2002.

Government Rights Statement

[0002]   This invention was made with federal government support from the National Institutes of Health of the U.S. Department of Health and Human Services under Grant No. A129329 to the City of Hope. The United States government may have certain rights in the invention.

FIELD OF THE INVENTION

[0003]   The present invention relates to RNA interference (RNAi), and more particularly to expression systems for producing interfering RNA molecules, including small interfering RNA molecules (siRNAs) and siRNA precursors in mammalian cells.

BACKGROUND OF THE INVENTION

[0004]   Gene therapy remains a promising treatment for AIDS because of the limited effectiveness of existing antiviral agents such as immunotherapy, protease inhibitors and vaccines. Several types of RNA gene therapies have been developed and shown to inhibit HIV-1 replication in mammalian cell cultures; these include antisense RNA, catalytic RNA (ribozymes) and high-affinity RNA ligands

1

(apamers or decoys). However, their efficiencies appear to be highly variable and, in many cases, ineffective.

[0005] RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in animals and plants initiated by double-stranded RNA that is homologous in sequence to the silenced gene. (Sharp, P.A., 2001) This powerful genetic technology has recently been shown to be useful in mammalian cells (Elbashir, S.M., et al., 2001; Novina, C.D., et al., 2002; Brummelkamp, T.R., et al., 2002; Paddison, P.I., et al., 2002; Lee, N.S., et al., 2002). One form of double-stranded RNA, known as small interfering RNA (siRNA), has been shown to target and inhibit expression of HIV-1 genes. (Lee, N.S., et al., 2002)

[0006] It is known that dsRNA $\geq$ 30 bp can trigger interferon responses in mammalian cells that are intrinsically sequence-nonspecific to the inducing dsRNA. (Elabashir, S.M., et al., 2001) Duplexes of 21-nucleotides (siRNAs) with overhanging 3' ends can mediate RNAi in a sequence-specific manner in cultured mammalian cells. (Elbashir, S.M., et al., 2001)

[0007] In mammalian systems, the sequence-specific RNAi effect can be observed by introducing siRNAs into target cells directly by transfection (Elbashir, S.M., et al., 2001 and Novina, C.D., et al., 2002), or indirectly by endogenous expression of siRNAs or short hairpin siRNAs (shRNAs). (Brummelkamp, T.R., et al., 2002; Paddison, P.I., et al., 2002; and Lee, N.S., et al., 2002). The stem-loop parts of the shRNAs parallel the naturally occurring stem-loop structures that are processed by the Dicer to yield small temporal RNAs (stRNAs) or microRNAs (miRNAs). Therefore, expression of shRNAs or miRNA precursors corresponding to

2

HIV-1 sequences could be acted upon by the human Dicer to yield HIV-specific siRNAs. Recently, expression of several shRNAs having different stem-loop structures from DNA templates in mammalian cells silenced several targets as effectively as synthetic siRNAs. (Brummelkamp, T.R., et al., 2002; and Paddison, P.I., et al., 2002) However, it remains unclear what makes the best RNA silencing hairpin.

[0008] Inhibition of HIV-1 replication using synthetic siRNAs or siRNAs expressed from plasmid DNAs has shown that siRNA technology may be useful as a therapeutic strategy to inhibit HIV-1 replication and infection in host cells. (Novina, C.D., et al., 2002; Paddison, P.I., et al., 2002; and Jacque, J.-M., et al. 2002) These data provide a basis for investigating therapeutic uses of siRNAS as anti-HIV-1 agents. For this purpose, siRNAs are expressed in cells to confer immunity to HIV-1 infection and prevent viral replication.

[0009] One approach for expressing siRNAs in cells involves inserting siRNA genes into a viral vector to be transduced into primary cells. Vector-based strategies that target combinations of viral genes and/or cellular genes should provide an improvement to RNA-based antiviral therapeutics. CCR5, an HIV-1 co-receptor, provides an attractive target candidate because homozygous deletions in CCR5 effectively confer protection from HIV-1 without any serious deleterious effects in immune functions in humans. A vector-based strategy for silencing HIV-1 *rev* in human cells has been demonstrated using the U6 promoter system. (Paddison, P.I., et al., 2002).

[00010] The basic design of the VA1 vector is described in Cagnon and Rossi, <u>Antisense and Nucleic Acid Drug Development</u> 10:251-261, 2000, incorporated herein by

3

reference. The use of the adenoviral VA1 promoter for ribozyme expression is described in U.S. Patent No. 6,100,087 (Rossi et al.), which also is incorporated herein by reference.

[00011] Vector-based siRNA-directed gene silencing in primary cells is lower than that seen in cell lines possibly because of poor expression of siRNAs or lower efficiency of silencing machinery in primary cells.

[00012] Strategies are needed to improve the expression and efficacy of silencing RNAs in primary cells. Strategies include alternative promoter systems for expressing interfering RNA molecules, and particularly siRNAs, in primary cells. The present invention addresses this need.

SUMMARY OF THE INVENTION

[00014] In one aspect, the present invention provides a VA1 Pol III promoter system, and preferably an adenoviral VA1 promoter system, for expressing interfering RNA (RNAi) molecules, including siRNA and siRNA precursor molecules, in a cell, preferably a mammalian cell. One advantage of this promoter system is that the VA1 transcript is transported to the cytoplasm, which is where the RNAi associated enzyme Dicer is localized.

[00015] In a preferred embodiment, a construct encoding an RNAi molecule is embedded in a non-essential stem region of the VA1 promoter gene. The RNAi molecule is expressed as a double stranded RNA, which may include a shRNA or other siRNA precursor that can serve as a substrate for the enzyme Dicer, which digests the RNA into an about 21-23 nucleotide duplex that can function as an siRNA.

[00016] In another preferred embodiment, the VA1 gene contains a construct encoding a precursor microRNA (miRNA),

4

which may provide higher expression levels or efficacy in primary cells. miRNA precursors likely are processed by the enzyme dicer into an about 21-24 nucleotide siRNA. In a more preferred embodiment, human mir-30 and let-7a-3 miRNAs may be utilized because of their RNAi effect in human cells.

[00017]   In another aspect, the present invention provides methods for expressing RNAi molecules in mammalian cells using the above-described adenoviral VA1 Pol III promoter system. The methods are useful for inhibiting gene expression and/or replication.

[00018]   In a further aspect, the invention provides a mammalian cell, preferably a primary cell, into which has been introduced a VA1 promoter system containing a construct encoding an interfering RNA molecule. The construct and VA1 promoter gene preferably are operatively linked.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019]   Figure 1A is a schematic diagram of an RNA Pol III-based adenoviral VA1 expression construct in accordance with the invention. The diagram depicts the VA1 gene and insertion site for an siRNA or siRNA precursor construct.

[00020]   Figure 1B [SEQ ID NO: 1] depicts an siRNA hairpin precursor (shRNA) targeted to an HIV *rev* site II, which can be inserted at the BstEII site of a VA1 gene.

[0021]   Figures 2A-C are schematic diagrams of a VA1 expression cassette, precursor siRNA constructs, and VA1 promoter-siRNA precursor expression products.

[00022]   Figure 2A shows that miRNA encoding sequences are inserted at the BstEII site of the VA1 gene. The pol III promoter is represented by the A and B boxes.

[00023]   Figure 2B shows two preferred human miRNA constructs (gugaagccacagaug [SEQ ID NO: 2], uggggcucug

5

cccugcuaug ggau [SEQ ID NO: 3]).

[00024] Figure 2C shows expressed VA1 RNA (from Mfold version 3.0) containing miRNA.

[00025] Figure 3 is a photograph of results of Northern gel analyses of expression of VA1-siRNA constructs in accordance with the invention. The lanes are left to right: U6 siII, U6si-irrelevant, VAsh-siteII with 8 base loop, VAsh-site II inverted (antisense-loop-sense) with 8 base loop, VAshII inverted with 9 base loop, VAshII inverted with 4 base loop, DNA oligo size marker. The 23 nucleotide transcript from the U6 promoter siRNAII is indicated. Full length, intermediate processed and siRNA processed products also are depicted.

[00026] Figure 4 is a graph showing the results of co-transfecting chimeric VA1 shRNA constructs with HIV-1 pNL4-3 proviral DNA into human 293 cells. The various VA1-shRNA chimeric constructs are indicated along with the positive control U6 siRNA expression construct. The letters F and R refer to the orientation of the shRNA sequence in the VA1 vector with respect to the transcription start point. F=forward sense-loop-antisense and R=reverse antisense-loop-sense. The loop sizes are also indicated.

DETAILED DESCRIPTION OF THE INVENTION

[00027] In the present invention, an RNA polymerase III (Pol III)-based adenoviral VA1 promoter gene (e.g., Figure 1A) is used to control expression of interfering RNAs, including siRNAs and siRNA precursors, in mammalian cells and preferably primary cells.

[00028] The VA1 Pol III promoter is able to tolerate variability in the sequences separating the two promoter control elements. Silencing RNA encoding sequences can be

6

inserted within the coding region, preferably stem-loop IV, of the VA1 gene, enabling silencing RNAs to be efficiently transcribed and stabilized by the secondary and tertiary structures of the natural RNA. VA1 transcripts are also primarily located in the cytoplasm where essential siRNA machinery such as silencing complexes (RISC) and Dicer are located. The cytoplasmic co-localization of the VA1 RNA with the siRNA machinery may improve the efficiency of siRNA-mediated RNAi.

[00029] In a preferred embodiment, miRNA precursors are appropriate silencing RNAs for cloning into VA1 RNA because they are likely processed by Dicer into about 21-24 nucleotide siRNAs (Fig. 2B). Among the many human miRNAs, natural and designed mir-30 miRNAs can inhibit the expression of target mRNAs in human cells. (Zeng, Y., et al. 2002). Human mir-30 and let-7a-3 miRNAs (having similar structure to mir-30) are useful for this purpose because of their RNAi effect in human cells. The constructs encoding miRNA precursors, containing the duplex sequences of the *rev* or CCR5 mRNA, can be designed and produced by PCR using DNA forward and reverse oligomers.

[00030] Before cloning into the VA1 vector, the designed mir-30 or let7 *rev* or CCR5 miRNA precursor constructs can be transcribed *in vitro* using DNA oligomer templates and T7 megashortscript kit (Ambion) to test their susceptibility to Dicer. Transcription templates are designed that contain T7 promoter sequences at the 5' end. The T7 transcribed miRNAs then are incubated with human Dicer and the products are visualized in a PAGE gel.

[00031] To determine whether Dicer is involved in RNAi *in vivo*, Dicer activity can be depleted from the transfected cells with Dicer siRNAs.

7

[00032] In a preferred embodiment, the miRNAs that are siRNA targeted against *rev* and/or CCR5 mRNA are cloned into the VA1 vectors using *Bst*EII sites (Fig. 2A). The constructs as well as control constructs are co-transfected into 293/EcR cells with the pIND-*rev*-EGFP or pIND-CCR5-EGFP target constructs. Inhibitory efficacy is monitored using assays, including FACS and fluorescent microscope imaging.

[00033] The intracellular localization of miRNA-VA1 constructs can be examined using *in situ* hybridization. (Lee, N.S., et al., 1999) A modified, Cy3-labeled DNA probe complementary to the insert in the VA1 cassette and a FITC-labeled DNA probe complementary to the target can be used for this purpose. Appropriate controls complementary to intracellular sequences (U3 for the nucleolus, U6 for the nucleus, and tRNA lys for the cytoplasm) also can be used along with a third fluorochrome to confirm the intracellular compartment. Following preliminary identification of effective shRNA constructs, they are expressed in human CD34+ cells and T-cells challenged with HIV-1.

[00034] Resistance to siRNAs can and will occur in HIV-1 infected individuals. To circumvent this problem, vectors can be produced that simultaneously express multiple siRNAs targeted to different sites in the virus, preferably along with an siRNA targeted against the cellular co-receptor CCR5.

[00035] Before gene therapy, the anti-HIV-1 candidate constructs are effectively transduced into primary cells or quiescent stem cells. Candidate constructs can be subcloned into a vector, examples of which are described in co-pending U.S. Application Serial No. 10/365,643, filed February 13, 2003, incorporated herein by reference. Candidate constructs are subcloned preferably into a lentiviral

8

vector, and more preferably an HIV7 lentiviral vector. The HIV7 lentiviral vector contains the polypurine tract of HIV-1 for enhanced integration and the woodchuck heptatitis post-transcriptional regulatory element (WCRE) for enhancing RNA stability. The 5' LTR is replaced in the vectors with the CMV promoter that subsequently is eliminated on reverse transcription of the viral RNA. In the final construct, the Pol III cassette is inserted downstream of the polypurine tract and Rev response element (RRE) and immediately upstream of the CMV promoter in pHIV-7. The EGFP gene also can be inserted into the vector to provide a selection marker.

[00036]   In a preferred embodiment, the vectors harboring VA1 miRNAs are packaged by co-transfecting them along with pCMV-G expressing the VSV G protein, pCHGP-2 expressing the HIV-1 Gag and Pol proteins, and pCMV-Rev. CEM cells are used to pre-screen the expression levels of the constructs prior to their expression in primary cells. Using supernatants from transfected 293T cells, the CEM cells are transduced and the expression levels of lentiviral vectors harboring VA1 miRNAs are analyzed using Northern gel analyses.

[00037]   The transductants then are infected with lab strains of HIV-1 such as M-tropic JRFL and BaL and T-tropic IIIB and NL4-3. Suppression of viral replication is determined by monitoring RT and p24 levels, syncytia formation, and survivability. Once the constructs are determined to function properly, they are then transduced into primary CD34+ cells and T-lymphocytes using, for example, the protocol by Bauer et al. (Bauer, G. et al., 1997) Transduced cells then are evaluated for siRNA expression and anti-HIV-1 activities.

[00038]   The present invention is further detailed in the
following Examples, which are attached by way of
illustration and are not intended to limit the invention in
any manner.

Example 1

[00039]   VA1-miRNAs and VA1-siRNAs can contain 1) 21-nt
sense, 2) 4, 8, or 9 base loop for siRNA or miRNA (mir-30 or
let-7a-3), and 3) 21-nt antisense strand complementary to a
HIV-1 rev target (total ~50-nt).   To make inactive mutant
mi- or si-RNA variants, 4-nt in the middle of the stem
sequences were mutated to be non-complementary to the
target.   *Bst*EII restriction fragments containing mi- or si-
RNA sequences are prepared from synthetic oligonucleotides,
which share 12 bases of complementary sequence at their 3'
end.   These were annealed and the primer ends extended using
Taq polymerase and several rounds of PCR.   The wt and mt mi-
or si-RNA sequences are cloned into the *Bst*EII site of pVA1
(Fig.1A).   The sequences of the loops are: 4 base loop: TTAA
[SEQ ID NO: 4], 8 base loop: G̲A̲A̲G̲C̲T̲T̲G [SEQ ID NO:
5](underlined: HindIII site), and 9 base loop: TTCAAGAGA
[SEQ ID NO: 6].

Example 2

[00040]   VA1-siRNA expression constructs targeting *rev* site
II were co-transfected with a *rev*-EGFP fusion construct.
(Figure 3)   The various VA1 shRNA chimeric constructs or a
U6 promoter siRNA expression construct were transfected
using a cationic lipid into human 293 cells.   Forty eight
hours after transfection, RNAs were isolated from the cells
and electrophoresed in a 6% denaturing polyacrylamide gel.
The RNAs were electroblotted to a nylon membrane and

hybridized with a ^{32}P labeled synthetic DNA oligonucleotide complementary to the antisense sequence of the shRNA or siRNA.

Example 3

[00041]   Several different loop sizes and the relative orientations of the sense and antisense strands were tested by co-transfecting VA1-siRNA constructs with pNL4-3 in human 293 cells.   Plasmids encoding the various constructs were co-transfected with HIV-1 proviral DNA into human 293 cells and HIV-1 p24 assays were carried out on samples taken at the times indicated.   The results (Figure 4) demonstrate that an 8 base loop was among the most effective at generating a functional siRNA, and that the relative orientation of sense and antisense (#1 and #2) do not appear to affect the efficacy of the siRNAs.

[00042]   The publications and other materials used herein to illuminate the background of the invention, and provide additional details respecting the practice of the invention, are incorporated herein by reference as if each was individually incorporated herein by reference.

[00043]   While the invention has been disclosed in this patent application by reference to the details of preferred embodiments of the invention, it is to be understood that the disclosure is intended in an illustrative rather than in a limiting sense, as it is contemplated that modifications will readily occur to those skilled in the art, within the spirit of the invention and the scope of the appended claims.

Listed References

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